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Cancer and Stroma-Targeted Immunotherapy with a Genetically Modified DC Vaccine

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14. ABSTRACT

While current DC vaccines are safe, their antitumor activity is limited. This is foremost due to the presence of regulatory T cells (Tregs), which create an immunosuppressive environment in breast cancer patients. In addition, there is increasing evidence that effective solid tumor vaccines have to target cancer cells as well as their supporting stroma. Thus, overcoming Treg mediated immune suppression and targeting the tumor stroma in addition to breast cancer cells may produce the desired increase in antitumor activity of DC vaccines for breast cancer. Recombinant lentiviral vector expressing A20-shRNA, HER2 and FAP was prepared and 4T1.2-neu tumor bearing mice were immunized with the lentivirus transduced DC vaccine. We found the DC vaccine induced robust T cell responses against HER2 and FAP, resulting in enhanced antitumor effect. Thus, the DC vaccine that target not only HER2, but also Treg and FAP, might present the optimized DC vaccine strategy against HER2+ breast cancer.

15. SUBJECT TERMS
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Introduction

The intent of this project is to develop an effective DC vaccine strategy for patients with breast cancer. While current DC vaccines are safe, their antitumor activity is limited (1-3). This is foremost due to the presence of regulatory T cells (Tregs), which create an immunosuppressive environment in breast cancer patients (4-11). In addition, there is increasing evidence that effective solid tumor vaccines have to target cancer cells as well as their supporting stroma (12-20). **Therefore, we hypothesized that overcoming Treg mediated immune suppression and targeting the tumor stroma in addition to breast cancer cells may produce the desired increase in antitumor activity of DC vaccines for breast cancer**.

In our previous study, we have demonstrated that silencing the zinc-finger A20 in DCs overcomes Treg-mediated immunosuppression in tumor bearing mice using ovalbumin as a model antigen (21). Since effective Treg depletion prior to vaccination has proven difficult, A20-silenced DC vaccine might represent an alternative strategy to overcome Treg-mediated immunosuppression. Thus in this project we proposed to develop an A20-silenced DC vaccine to activate breast cancer- and stroma-specific cytotoxic T lymphocytes (CTL), while concomitantly inhibiting Tregs. This novel vaccine targets the human epidermal growth factor receptor (HER)2 expressed on breast cancer cells and the fibroblast activating protein (FAP) expressed on the supporting tumor stroma.

In the first year of this award, recombinant lentiviral vector expressing shA20, FAP, and HER2 (Lv-shA20-FAP-HER2) was prepared and immunized to 4T1.2-neu tumor bearing mice. First, Lv-shA20-FAP-HER2 and control vectors (Lv-shA20-FAP, Lv-shA20-HER2, Lv-shGFP-FAP, Lv-shGFP-HER2, Lv-shGFP-FAP-HER2) were prepared and transduced to mice bone-marrow derived DCs. Then these DC vaccines were immunized to Balb/c mice. The results indicated that immunization of A20-silenced DC vaccine induced more robust T cell responses against FAP or HER2, compared to that of GFP-silenced DC vaccine. In addition, the immunization of DC-shA20-FAP-HER2 inhibited 4T1.2-neu growth more efficiently, compared to that of control vaccines.
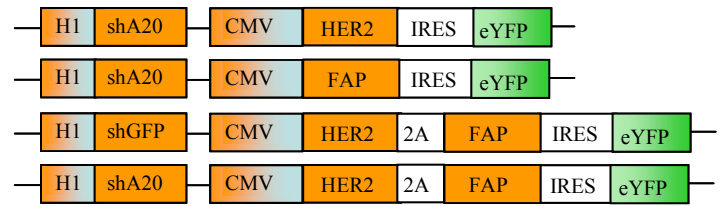
In the second year of this award, we have proposed to evaluate these DC vaccines using HER2 transgenic mice. However pilot studies shown FAP-targeted DC vaccine had limited therapeutic effects on tumor-bearing HER2-transgenic mice and it takes too long time to raise sufficient amount HER2 transgenic mice for further mechanistic studies. Therefore, we have focused on the mechanistic studies in an alternative B16 tumor model. The central hypothesis of this study is that co-targeting tumor antigen and FAP would more efficiently induce antigen spreading, resulting in the elimination of antigen loss variants. The results indicated that the immunization of DC-shA20-FAP-TRP2 significantly induced activation of CTLs against other tumor antigens expressed by B16. Further, the immunization of DC-shA20-FAP-OVA significantly inhibited B16 (antigen loss variant of B16-OVA) growth in mice, suggesting that co-targeting cancer antigen and FAP represents an optimized vaccination strategy.

Body

Task 1: To construct lentiviral vector coexpressing A20-shRNA, HER2, and FAP and evaluate the potency of DC-A20-shRNA-HER2-FAP to induce HER- and FAP-specific CTL and Th responses in 4T1.2-Neu bearing mice.

Preparation of animal protocol. Animal protocol AN3288 of "Immunization by DC vaccine" was amended to include the immunization of DC-shA20-FAP-HER2 and other control DC vaccines for the study and approved by Baylor College of Medicine.

Generation of recombinant lentiviral vector expressing A20-shRNA, HER2 and FAP. The recombinant lentiviral vectors expressing A20-shRNA, HER2 and FAP, including Lv-shA20-HER2-FAP, Lv-shGFP-HER2-FAP, Lv-shA20-HER2, Lv-shA20-FAP, Lv-shGFP-HER2, Lv-shGFP-FAP, and Lv-shA20 were prepared. We generated an expression cassette containing A20 shRNA, HER2 and FAP by PCR cloning (**Fig.1**). The lentiviral vector used in this study was pTRIPΔU3 CMV eGFP (21-24), which is self-inactivating (SIN vectors) with a 400 bp deletion in the U3 region of the 3' long terminal repeat (LTR), and contains a 178-bp fragment encompassing the central polypurine tract (cPPT) and the central termination sequence (CTS). pTRIP-H1-BY-W vector was generated from pTRIPΔU3CMV eGFP for expression of siRNA from the H1 RNA promoter

Fig. 1: *Scheme of Expression cassettes encoding shA20, FAP, and HER2*

and co-expression of a bicistronic blasticidin resistance/eYFP selection marker. Murine FAP gene was kindly provided by Dr. J. Cheng (FCCC, PA). Recombinant Lentiviral vectors were generated by standard methods established in our laboratory. All vectors were verified by DNA sequencing. Recombinant pseudotyped lentiviral vectors were generated by co-transfection of three plasmids into 293 cells and concentrated by ultracentrifugation, as described previously.

Characterization of genetically modified DC functions *in vitro*. We transduced bone-marrow derived DCs from Balb/c mice with Lenti-shA20-HER2-FAP, or controls. Mouse bone marrow (BM)-derived DCs were prepared as described in our previous study (21-24). Briefly, mouse bone marrow was flushed from limbs, passed through a nylon mesh, and depleted of red cells with ammonium chloride. After extensive washing with RPMI-1640, cells were cultured with 2.5 ml of RPMI-1640 supplemented with 10% FBS, mGM-CSF/ml (20 ng/ml) and recombinant mouse IL-4 (20 ng/ml; PeproTech). On days 2 and 4 of culture, the supernatant was removed and replaced with fresh media containing 20 ng/ml of rmGM-CSF and 20 ng/ml of rmIL-4. All cultures were incubated at 37°C in 5% humidified CO_2. Nonadherent granulocytes were removed after 48 hr of culture and fresh medium was added. After 7 days of culture, >80% of the cells expressed characteristic DC-specific markers as determined by FACS. Transduction of BM-derived DCs with these LV vectors routinely yielded 30-60% of cells (data not shown). A20 expression in Lv-shA20 transduced DC was downregulated by 80-90% in comparison to DCs transduced with the control vector (data not shown). FAP and HER2 expression of transduced BM-DCs was confirmed by RT-PCR.

Evaluation of the potency of the resultant vaccine to induce HER2- and FAP-specific CTL responses in 4T1.2-neu bearing mice models. *shA20-DC vaccines induce potent FAP- or HER2-specific immune response.* We tested whether A20-silenced DC vaccine
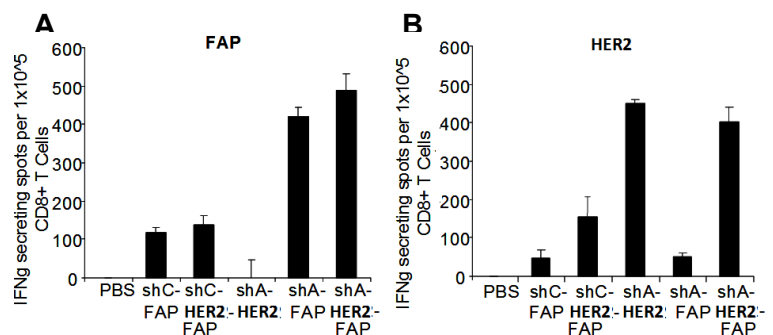
Fig. 2. ELISPOT assays of T-cell responses against FAP and HER2. *Mice were immunized with DC vaccines or PBS. Isolated CD8^{+} T cells from immunized mice (2-3/group) were subjected to IFNγ ELISPOT assays against FAP **(A)** and HER2 **(B)***

strategy was able to induce enhanced FAP- and HER2-specific T cell responses. Balb/C mice were immunized with DC vaccines or PBS, and the functional status of CD8$^+$ and CD4$^+$ T cells was then evaluated using interferon (IFN)-γ ELISPOT assays. We observed that mice immunized with A20-silenced DC vaccines had significantly higher frequencies of IFN-γ$^+$ CD8$^+$ T (**Fig. 2)** and CD4$^+$ T cells (Not shown), compared to those given DC-shGFP vaccines**.** These data demonstrate a critical role for A20 in control of the magnitude of FAP- and HER2-specific T-cell responses.

DC-shA20-FAP-HER2 vaccination inhibits 4T1-neu progression. We investigated whether DC-shA20-FAP-HER2 may induce more potent anti-stroma and anti-tumor immunity with the ability to control the growth of tumors. DC vaccines were immunized to the Balb.C mice followed by inoculation of 4T1.2-neu tumor in the fourth mammary fat pad in 7 days later (**Fig. 3**). By targeting both FAP and HER2, one immunization of DC-shA20-FAP-HER2 completely prevent the progression of 4T1.2-neu tumor in 80 days. These results demonstrate the DC-shA20-FAP-HER2 has potent antitumor activity.

Fig.3: Comparison of antitumor activity. Mice were immunized with 1x10^6 DCsor PBS control followed by inoculation of 4T1.2-neu cells (5x10^5) 7 d later .
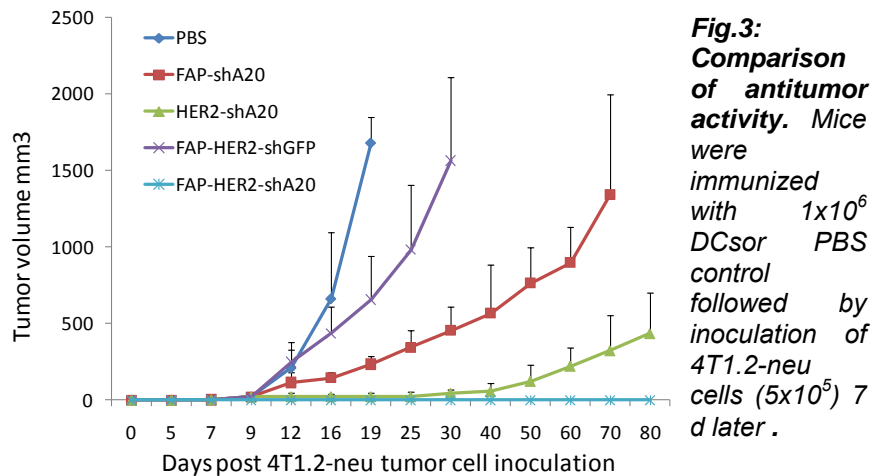
DC-shA20-FAP vaccine decreases immunosuppressive cells in the tumor. We investigated whether DC-shA20-FAP vaccination results in a decrease in suppressive cells in the tumors. While we are evaluating our vaccine using 4T1.2-neu mice model, we also used B16 mice model. Staining B16 tumors for CD11b identified a large population of infiltrating macrophages (**Fig.4A**), which significantly decreased after vaccination of FAP-targeted DC vaccine. We observed that both the CD11b$^+$Gr1^{dim} population and CD11b$^+$Gr1^{hi} population were significantly decreased after vaccination of FAP-targeted DC vaccine from 41.38% to 26.44% and 4.37% to 2.48%, respectively. In addition, upregulation of CD80 expression on tumor infiltrating CD11c$^+$ population was observed in the recipients of FAP-targeted DC vaccine (**Fig.4B**). Taken together, these data indicate that our FAP-targeted DC vaccine decreases immature DC and tumor-associated macrophages, supporting our hypothesis in Aim 1 that the A20-silenced FAP-targeted DC vaccine has the potential to reverse the suppressive tumor microenvironment.
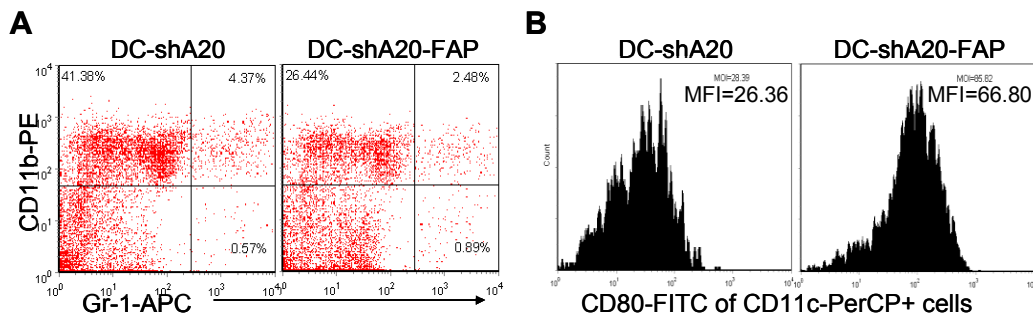
*Fig.4. Influence of FAP-targeted DC vaccine on accumulation and maturation of macrophages and dendritic cells. C57BL/6 mice were inoculated s.c. with B16 cells. When the tumor sizes reached >5mm in diameter, mice were immunized with 1x10^6 DC-shA20-FAP, or DC-shA20. At 7 days after vaccination, tumor infiltrating cells were subjected to flow analysis. **A.** Dot plots show the effect of DC-shA20-FAP on the proportion of CD11b$^+$Gr1^{dim} and CD11b$^+$Gr1^{hi} cells. **B.** Histograms show CD80 expression in CD11c$^+$ gated tumor infiltrating cells with the MFI of CD80 indicated.*

DC-shA20-FAP vaccine enhances CD8 infiltration in the tumor. We also investigate whether DC-shA20-FAP vaccine enhance CD8 infiltration in B16 tumors. Staining for CD8 showed that there was a dramatic

increase in CD8+ T cells within the tumor after DC-shA20-FAP vaccination. These changes were statistically significant and represented a 3-fold (7.72%:19.31%) change in CD8+ proportion in the tumor (**Fig. 5**). These data support our hypothesis in Aim.1.C that A20-silenced FAP-targeted DC vaccine has the potential to enhance the infiltration and proliferation of tumor-specific T cells at the tumor site.

Fig.5. DC-shA20-FAP enhances CD8+ T cell infiltration in the tumor. *C57BL/6 mice were inoculated s.c. with B16 cells. When the tumor sizes reached >5mm in diameter, mice were immunized with DCs. 7 days later, tumor infiltrating cells were subjected to flow analysis.*

Summary. The data described above demonstrated that DC-shA20-FAP-HER2 vaccine induce robust T cell responses against FAP or HER2, resulting enhanced antitumor effect.

Task 2: To dissect the mechanisms for the enhanced anti-tumor potency of DC-A20-shRNA-HER2-FAP.

Functional characterization of lentiviral vectors encoding shA20, FAP and TRP2. To evaluate the vaccine in a B16 model, we first constructed 4 lentiviral vectors encoding shA20 and FAP (Lv-shA20-FAP), shA20 and TRP2 (Lv-shA20-TRP2), shA20, FAP and TRP2 (Lv-shA20-FAP-TRP2), or a control shRNA, FAP, TRP2 (Lv-shCo-FAP-TRP2) (**Fig. 6A**). Transgene expression, and silencing of A20 was confirmed by RT-PCR in bone-marrow derived DCs transduced with VSV-G pseudotyped lentiviral vectors (**Fig. 6B,C**).
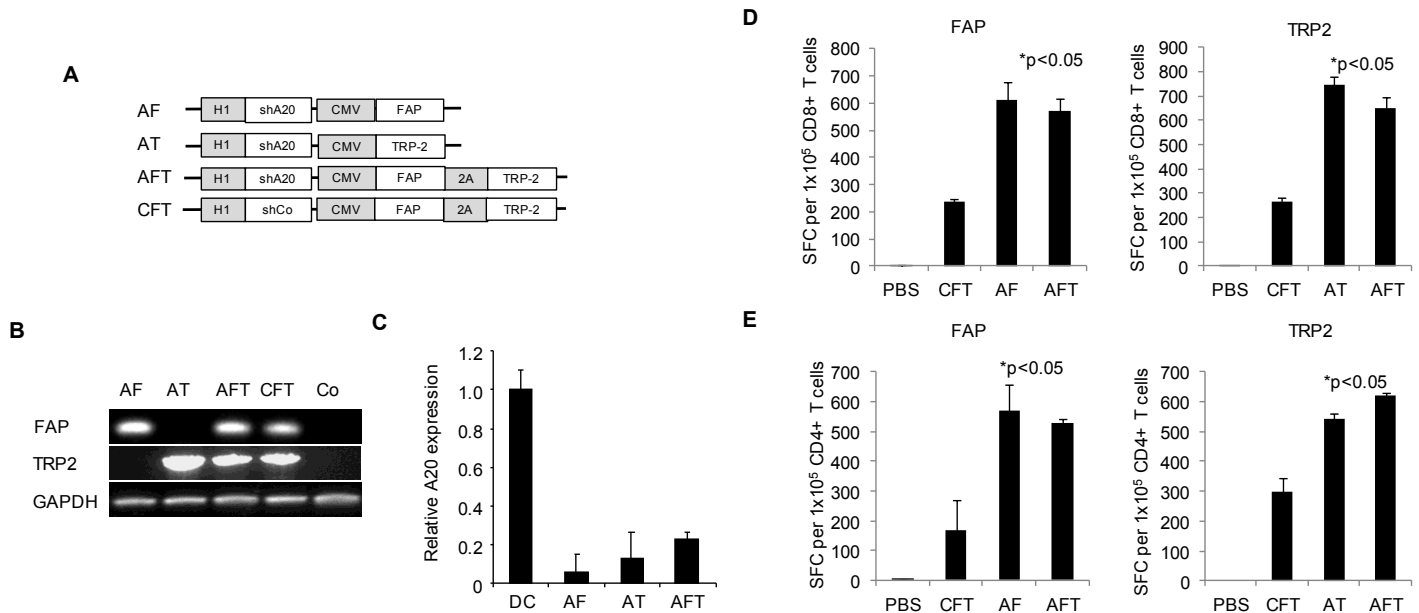
Fig.6.: Tumor- and FAP- co-targeted DC induce T-cell activation. *(A) Scheme of lentiviral constructs. (B and C) Mouse BM-DCs were transduced with lentivirus and FAP and TRP2 expression (B) and A20 expression (C) were detected by RT-PCR or Q-PCR individually. (D and E) Mice were immunized with 1 × 106 lentivirus-transduced BM-DCs in 25 μl sterile PBS or PBS control through footpad 14 days post vaccination splenocytes were prepared and CD8+ (D) and CD4+ (E) T cells selected. The frequency of FAP- and TRP2-specific T cells was determined using IFN-γ ELISPOT assays (n=2; assay performed in triplicates). AF, lentiviral vector coexpressing an A20-specific short-hairpin RNA (shRNA) and FAP; AT, lentiviral vector coexpressing A20-shRNA and TRP2; AFT, lentiviral vector coexpressing A20-shRNA, FAP, and TRP2; CFT, lentiviral vector coexpressing GFP-shRNA, FAP, and TRP2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PBS, phosphate-buffered saline.*

To demonstrate that Lv-transduced DCs induce FAP- and TRP2-specific T-cell responses, mice were vaccinated with 1x10⁶ DC-shA20-FAP, DC-shA20-TRP2, DC-shA20-FAP-TRP2, or DC-shCo-FAP-TRP2. Fourteen days post vaccination CD4- and CD8-positive splenocytes were isolated and the presence of FAP- and TRP2-specific T cells was determined by IFN-γ Elispot assays. Mice vaccinated with A20-silenced DC
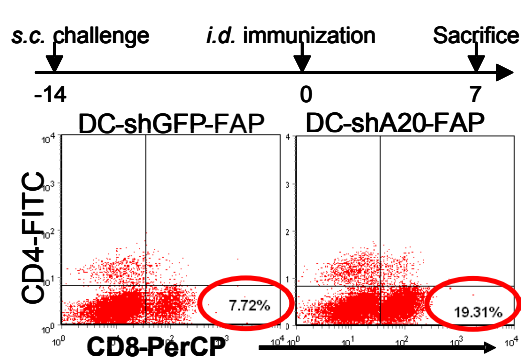
vaccines induced significantly higher FAP- and TRP2-specific CD4- and CD8-positive T-cell responses (p<0.05) than mice vaccinated with DC-shCo-FAP-TRP2, confirming our previous finding that silencing of A20 in DCs promotes the induction of robust CD4- and CD8-positive antigen-specific T-cell responses in vivo (**Fig. 6D,E**). There was no significant difference (p>0.05) in the induction of FAP- or TRP2-specific T cells responses after vaccinating mice with DCs expressing individual (DC-shA20-FAP or DC-shA20-TRP2) or both antigens (DC-shA20-FAP-TRP2), indicating absence of antigenic competition when both antigens are co-expressed.
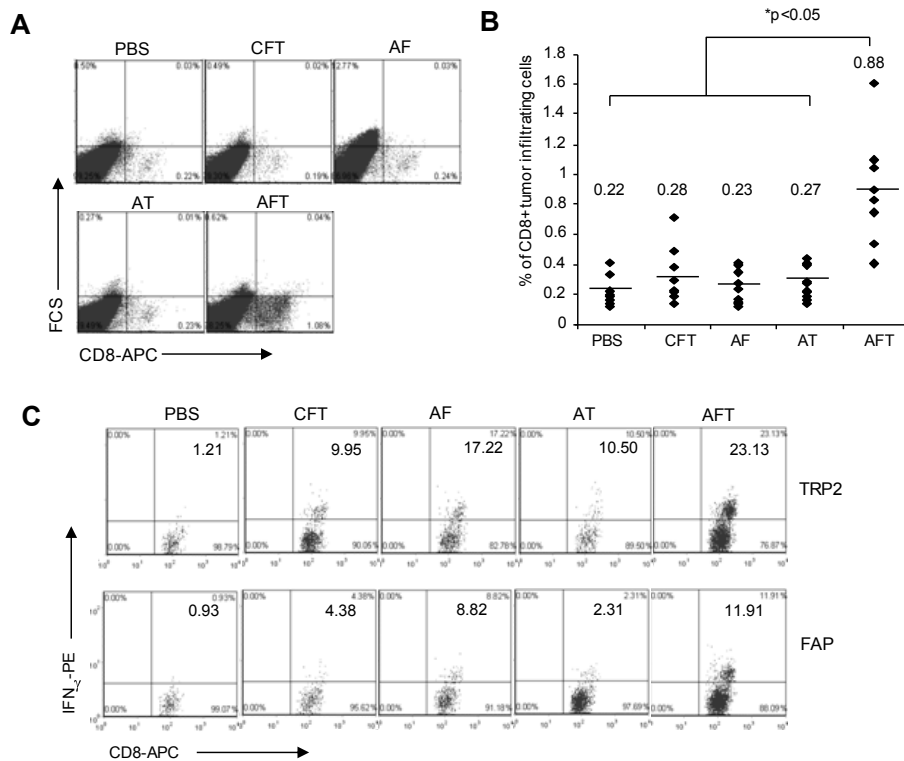
DC-shA20-FAP-TRP2 vaccine has potent antitumor activity. The B16 melanoma model is ideal to evaluate if targeting FAP-positive tumor stroma enhances antitumor effects since B16 cells do not express FAP. We confirmed the induction of FAP expression within 5 days post B16 tumor implantation by RT-PCR (**Fig. 7A**). Mice bearing 5 day-old B16 tumors were vaccinated with a single dose of the 1x106 DC-shA20-FAP, DC-shA20-TRP2, DC-shA20-FAP-TRP2, or DC-shCo-FAP-TRP2 vaccine. All A20-silenced vaccines had antitumor activity, while non-A20 silenced DC vaccine had none (**Fig. 7B**). There was no difference in the antitumor activity of DC-shA20-FAP and DC-shA20-TRP2 vaccines, indicating that T-cell responses against a stroma antigen alone can have significant antitumor effects (**Fig. 7B**). Targeting FAP and TRP2 simultaneously with the DC-shA20-FAP-TRP2 vaccine resulted in the greatest antitumor activity (DC-shA20-TRP2 vs DC-shA20-FAP-TRP2, p<0.05; DC-shA20-FAP vs DC-shA20-FAP-TRP2, p<0.05). This resulted in a significant increase in survival of mice that received DC-shA20-FAP-TRP2 vaccine in comparison to the other vaccine groups (**Fig.7C**).
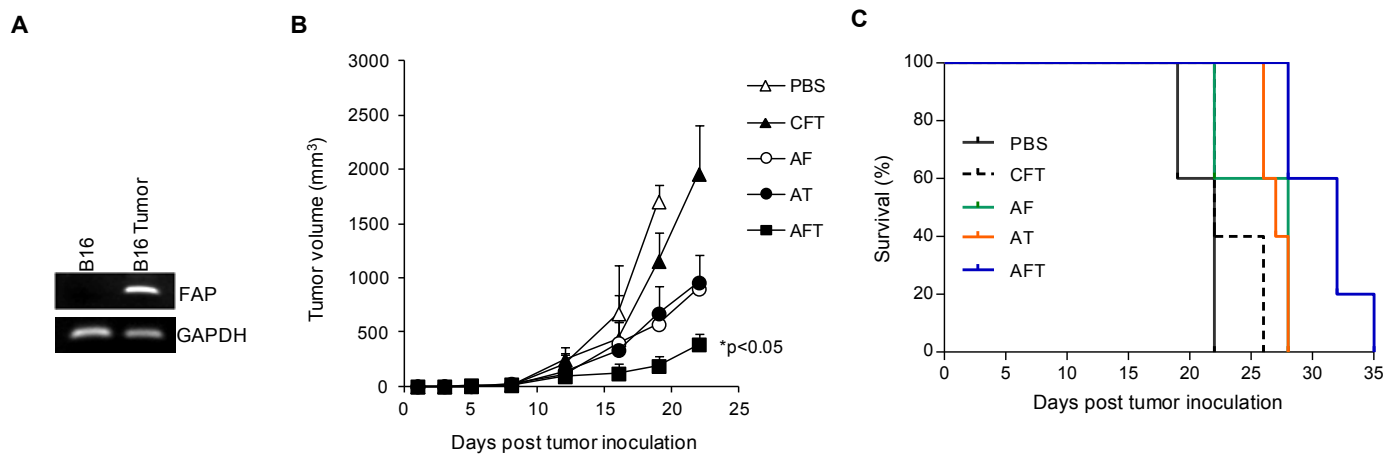
Fig.7.: DC-shA20-FAP-TRP2 vaccine has potent antitumor activity. (A) RT-PCR for FAP and GAPDH of B16 cell line, and day 5 B16 tumors. (B,C) Mice were inoculated with B16 followed by immunization with 1x106 DC-shA20-FAP (AF), DC-shA20-TRP2 (AT), DC-shA20-FAP-TRP2 (AFT), DC-shCo-FAP-TRP2 (ACT) or PBS on day 5 (n= 5 per group). (B) Cotargeting FAP and TRP2 with AFT resulted in the greatest antitumor response (AF vs AFT, p<0.05; AT vs AFT, p<0.05). (C) Kaplan-Meier survival curve (AF vs AFT, p<0.05; AT vs AFT, p<0.05).
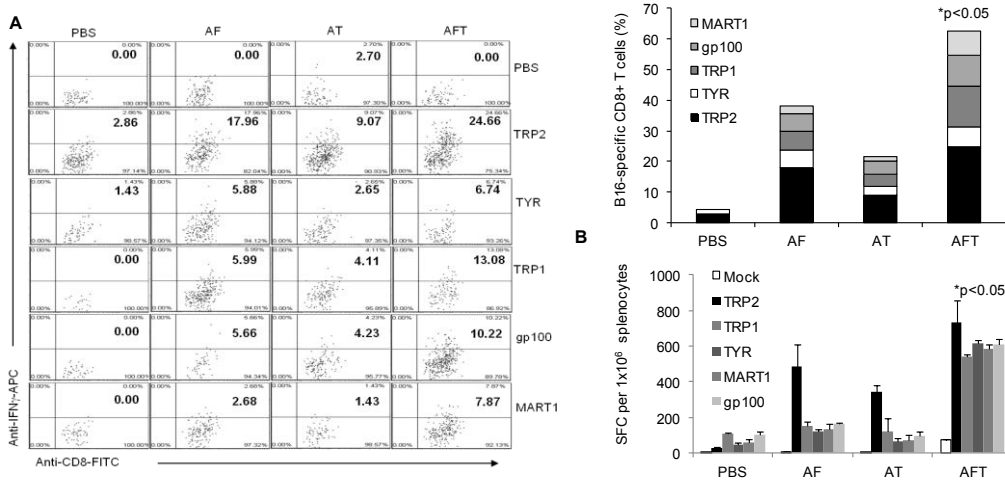
DC-shA20-FAP-TRP2 vaccine increases FAP- and TRP2-specific CD8-positive T cells in tumors. To investigate the mechanisms underlying the enhancement of the antitumor effects, we first determined the frequency of CD4- and CD8-positive T cells in B16 tumors 3 weeks post vaccination. While there was no significant difference in the percentage of CD4-positive T cells within tumors between groups of vaccinated mice (data not shown), there was a significant (p<0.05) 4-fold increase in the percentage of CD8-positive T cells after DC-shA20-FAP-TRP2 vaccination in comparison to control mice (**Fig. 8A,B**). In contrast, the monovalent vaccines or the non-silenced FAP/TRP2 DC vaccine did not increase the frequency of intratumoral CD8-positive T cells in comparison to controls. To investigate the specificity of the infiltrating CD8-positive T cells, tumor-infiltrating lymphocytes (TILs) were isolated 3 weeks post vaccination. Intracellular cytokine staining for IFN-γ was performed post stimulation with FAP- or TRP2-expressing DCs. DC-shA20-FAP-TRP2 vaccination induced the highest frequency of FAP- and TRP2-specific T cells in comparison to the other vaccines (**Fig. 8C**), mirroring the previously observed overall increase in the number of CD8-positive T cells within tumors.

A

PBS | CFT | AF
AT | AFT

FCS ↑
CD8-APC →

B

*p<0.05

% of CD8+tumor infiltrating cells

0.22   0.28   0.23   0.27   0.88

PBS   CFT   AF   AT   AFT

Fig.8.: DC-shA20-FAP-TRP2 vaccine increases FAP- and TRP2-specific CD8-positive T cells in tumors. *Mice were inoculated with B16 tumors followed by immunization with 1x106 DC-shA20-FAP (AF), DC-shA20-TRP2 (AT), DC-shA20-FAP-TRP2 (AFT) or PBS on day 5. Tumor tissues were dissected 3 weeks post vaccination (n=5). (A) Representative FACS analysis of infiltrating CD8+ T cells. (B) Summary data of all mice showing a significant increase (p<0.05) of CD8+ T cells in mice vaccinated with AFT. (C) TILs were restimulated with DCs transduced with FAP (DC-Lv-FAP) or TRP2 (DC-LV-TRP2) followed by intracellular staining of IFN-γ.*

C

PBS | CFT | AF | AT | AFT

TRP2: 1.21 9.95 17.22 10.50 23.13
FAP: 0.93 4.38 8.82 2.31 11.91

IFN-γ-PE ↑
CD8-APC →

DC-shA20-FAP-TRP2 vaccine induces antigen spreading. In the previous experiment we noticed that the DC-shA20-FAP vaccine induced TRP2-specific T-cell responses, suggestive of epitope spreading and the induction of antigen spreading. To further investigate this finding, B16 tumor-bearing mice were vaccinated on day 5 with DC-shA20-FAP, DC-shA20-TRP2, DC-shA20-FAP-TRP2, or PBS. After 3 weeks the frequency of T cells specific for the B16-associated tumor antigens tyrosinase-related protein 1 (TRP1), tyrosinase (Tyr), gp100, and melanoma-associated antigen recognized by T cells (MART1) among splenocytes and tumor infiltrating lymphocytes was determined. While all vaccines induced a significant increase of TRP1-, Tyr-, gp100-, and MART1-specific T cells in TILs in comparison to PBS-injected mice (**Fig. 9A**), the frequency of melanoma-specific T cells was highest in DC-shA20-FAP-TRP2 vaccinated mice (p<0.05). Only the DC-shA20-FAP-TRP2 vaccine induced systemic T-cell responses against TRP1, Tyr, gp100, and MART1 (**Fig.9B**). These results indicate that targeting of the tumor stroma as well as the tumor enables the induction of antigen spreading.

A

PBS | AF | AT | AFT

PBS: 0.00 0.00 2.70 0.00
TRP2: 2.86 17.96 9.07 24.66
TYR: 1.43 5.88 2.65 6.74
TRP1: 0.00 5.99 4.11 13.08
gp100: 0.00 5.66 4.23 10.22
MART1: 0.00 2.68 1.43 7.87

Anti-IFN-γ-APC ↑
Anti-CD8-FITC →

B

B16-specific CD8+ T cells (%)

□ MART1
■ gp100
■ TRP1
□ TYR
■ TRP2

*p<0.05

PBS   AF   AT   AFT

SFC per 1x10⁶ splenocytes

□ Mock
■ TRP2
■ TRP1
■ TYR
■ MART1
□ gp100

*p<0.05

PBS   AF   AT   AFT

Fig.9.: DC-shA20-FAP-TRP2 vaccine induces antigen spreading. *Mice were inoculated with B16 tumors followed by immunization with 1x106 DC vaccines or PBS on day 5. TILs and Splenocytes were prepared 3 weeks post vaccination. (A) TILs were restimulated with DC-Lv-TRP2, DC-Lv-TRP1, DC-Lv-TYR, DC-Lv-gp100, DC-Lv-MART1 or mock DCs followed by intracellular staining of IFN-γ. FACS analysis and a cumulative bar graph are shown. (B) Splenocytes were subjected to IFN-γ Elispot assays.*

Antigen spreading results in enhanced antitumor activity. Having shown that the developed compound vaccines induced B16 antigen spreading we wanted to determine if these antigen-specific T cells have anti-tumor activity. Mice were inoculated with B16-OVA and B16 on their right or left flanks, and after 5 days vaccinated with DC-shA20-FAP, DC-shA20-OVA, DC-shA20-FAP-OVA, or PBS. DC-shA20-OVA vaccination only inhibited the growth of B16-OVA, while the DC-shA20-FAP vaccine inhibited B16 as well as B16-OVA tumor growth (**Fig. 10A,B**). The DC-shA20-FAP-OVA vaccine had the greatest antitumor activity against both tumor cell lines (p<0.05; **Fig. 10A,B**). This is consistent with our observation that co-targeting tumor antigen and FAP induces systemic T cells responses against tumor antigen not present in the vaccine (Fig.4B). While DC-shA20-OVA vaccination did not result in an increase in overall survival, due to the antigen negative B16 tumors, DC-shA20-FAP or DC-shA20-FAP-OVA vaccination resulted in a significant increase in overall survival in comparison to DC-shA20-OVA (p<0.05; **Fig 10C**).
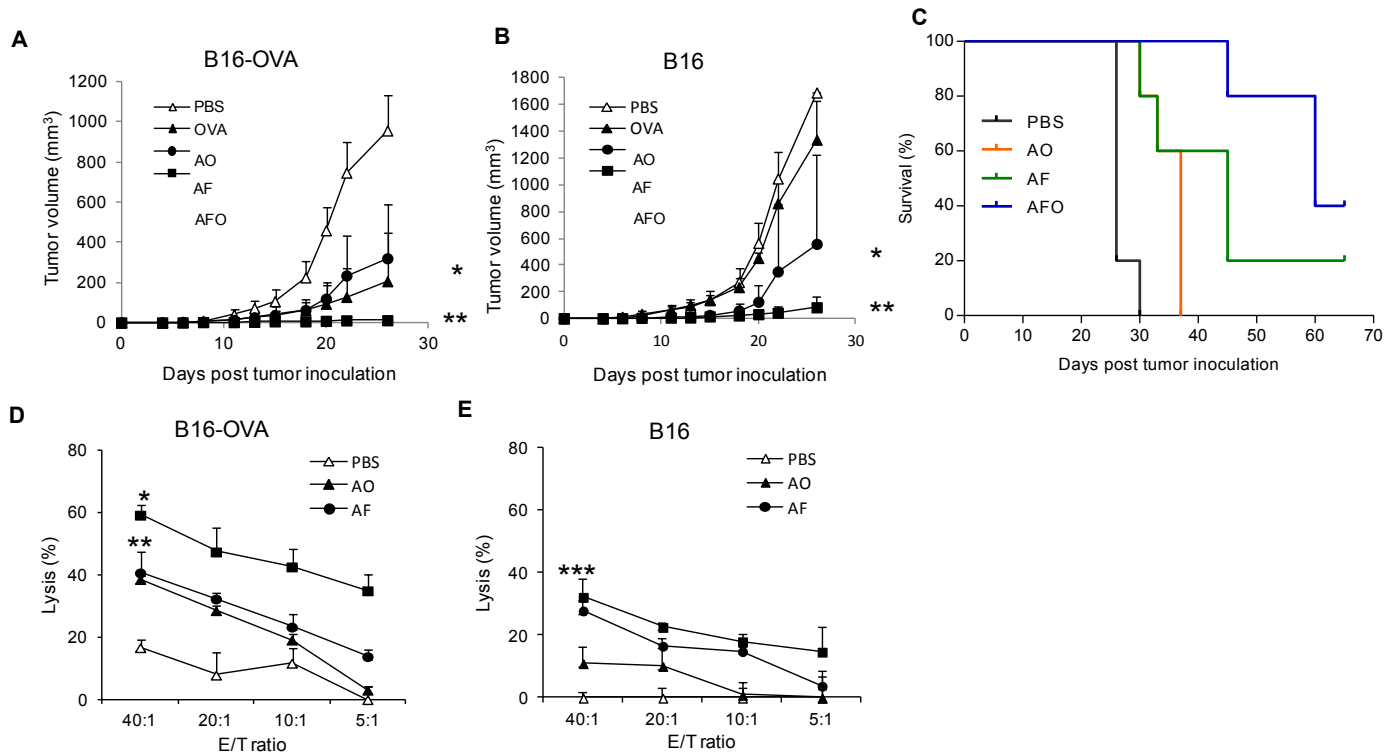
Figure10: Induced T cells specific for melanoma antigens not present in the vaccine have antitumor activity. (A-C) Mice were inoculated with B16-OVA or B16 tumor on the right or left flank separately followed by immunization with 1x106 DC-shA20-FAP (AF), DC-shA20-OVA (AO), DC-shA20-FAP-OVA (AFO) or PBS 5 days later (n= 5 per group). B16-OVA (A) and B16 (B) tumor growth was measured. The AFO and the AF vaccine had anti-B16 activity with AFO being superior (p<0.05). (C) Kaplan-Meier survival curve (AO vs AF, p<0.05; AO vs AFO, p<0.05). (D and E) Mice were inoculated with B16-OVA followed by immunization with 1x106 AF, AO, AFO, and PBS 5 days later. 3 weeks later, splenocytes were prepared and cultured in vitro in the presence of B16-OVA tumor lysate and IL2 for 5 days and their cytolytic activity was evaluated in standard subjected 51Cr release assay against B16-OVA (D) or B16 (E). Splenocytes isolated from AF and AFO vaccinated mice had significant cytolytic activity B16. *AFO vs AF and AO, p<0.05; **AF and AO vs PBS, p<0.05;***AFO and AF vs AO, p<0.05).

To further confirm the systemic induction of T cells specific for antigens not present in the vaccine, mice were injected with B16-OVA and vaccinated after 5 days with DC-shA20-FAP, DC-shA20-OVA, DC-shA20-FAP-OVA, or PBS. After 3 weeks splenocytes of vaccinated mice were isolated and restimulated for 5 days ex vivo before performing a cytotoxicity assay with B16-OVA and B16 cells as targets. Splenocytes harvested from DC-shA20-FAP or DC-shA20-FAP-OVA vaccinated mice showed significant killing of B16-OVA and B16 cells (**Fig. 10D,E**), where as the DC-shA20-OVA vaccine primarily induced OVA-specific T-cell responses.

Taken together, our results demonstrate that a DC vaccine in which the antigen presenting attenuator A20 is inhibited, and that targets FAP-positive CAFs and the tumor antigen has potent antitumor effects, enables cross-presentation of tumor antigens by intratumoral APCs resulting in the broad-based induction of T cells specific for tumor antigens not included in the vaccine.

Key Research Accomplishments

- Construction of lentiviral vector expressing mouse A20 shRNA, FAP, and HER2.
- Silencing of A20 in DCs could enhance FAP- and HER2-specific T cell immune responses.
- **Silencing of A20 in DCs could enhance anti-tumor effect in 4T1.2-neu bearing mice.**
- DC-shA20-FAP-TRP2 could enhance anti-tumor effect in B16 model.
- DC-shA20-FAP-TRP2 could induce antigen spreading in B16 model.
- **DC-shA20-FAP-OVA could eliminate antigen loss variants B16 in B16-OVA/B16 model.**

Reportable Outcomes

1. Presentation: An A20-silenced, stroma-targeted DC vaccine reverses the immunosuppressive tumor microenviroment resulting in potent antitumor activity. Zhu W, Zhou X, Rollins L, Rooney CM, Gottschalk S, Song XT. Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope Meeting, August 2-5, 2011. Orlando Marriott World Center Hotel, Orlando, FL.
2. Publication: Kakarla S, Song XT, Gottschalk S. Cancer-associated fibroblasts as targets for immunotherapy. Immunotherapy. 2012 Nov;4(11):1129-38. doi: 10.2217/imt.12.112. Review.
3. Manuscript: Stephen Gottschalk , Feng Yu, Minjun ji, Sunitha Kakarla, Xiao Tong Song. A vaccine that cotargets tumor cells and cancer associated fibroblasts induces bystander T cells with potent antitumor activity

Conclusions

- Demonstrates critical role for A20 in regulating the extent of T cell responses against FAP and HER2
- Demonstrates critical role for co-targeting FAP and tumor antigen in eliminating antigen loss variants.
- Providing an effective approach for developing potent DC vaccine against breast cancer.

References

1. Czerniecki, B.J., Koski, G. K.,Koldovsky, U.,Xu, S.,Cohen, P. A.,Mick, R.,Nisenbaum, H.,Pasha, T.,Xu, M.,Fox, K. R.,Weinstein, S.,Orel, S. G.,Vonderheide, R.,Coukos, G.,DeMichele, A.,Araujo, L.,Spitz, F. R.,Rosen, M.,Levine, B. L.,June, C.,Zhang, P. J. Targeting HER-2/neu in early breast cancer development using dendritic cells with staged interleukin-12 burst secretion. Cancer Res 67, 1842-1852 (2007).
2. Mittendorf, E.A., Peoples, G.E. & Singletary, S.E. Breast cancer vaccines: promise for the future or pipe dream? Cancer 110, 1677-1686 (2007).
3. Rosenberg, S.A., Yang, J.C. & Restifo, N.P. Cancer immunotherapy: moving beyond current vaccines. Nat Med 10, 909-915 (2004).
4. Campoli, M., Ferrone, S., Zea, A., Rodriguez, D.C., Ochoa, A.C. Mechanisms of tumor evasion. Cancer Treat Res, 28 (2005).
5. Kortylewski, M., Kujawski, M., Wang, T., Wei, S., Zhang, S, Pilon-Thomas, S., Niw, G., Kay, H., Mule, J., Kerr, W.G., Jove, R., Pardoll, F., Yu, H. Inhibiting stat 3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. Nat Med, 8 (2005).
6. Gillanders, W.E., Thalachallour, M. Progress in the development of a breast cancer vaccine: mammaglobin A as an attractive target for vaccine therapy. Breast Diseases: A Year Book Quarterly., 3 (2006).
7. Liyanage, U.K., Moore, T.T., Joo, H-G., Tanaka, Y., Hermann, V., Doherty, G., Drebin, J.A., Strasberg, S.M., Eberlein, TJ., Goedegebuure, P.S., Linehan, D.C. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinomas. J Immunol, 6 (2002).
8. Woo, E.Y., Chu, C. S.,Goletz, T. J.,Schlienger, K.,Yeh, H.,Coukos, G.,Rubin, S. C.,Kaiser, L. R.,June, C. H. Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. Cancer Res 61, 4766-4772 (2001).
9. Curiel, T.J., Coukos, G.,Zou, L.,Alvarez, X.,Cheng, P.,Mottram, P.,Evdemon-Hogan, M.,Conejo-Garcia, J. R.,Zhang, L.,Burow, M.,Zhu, Y.,Wei, S.,Kryczek, I.,Daniel, B.,Gordon, A.,Myers, L.,Lackner, A.,Disis, M. L.,Knutson, K. L.,Chen, L.,Zou, W. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 10, 942-949 (2004).
10. Wang, H.Y., Lee, D. A.,Peng, G.,Guo, Z.,Li, Y.,Kiniwa, Y.,Shevach, E. M.,Wang, R. F. Tumor-specific human CD4+ regulatory T cells and their ligands: implications for immunotherapy. Immunity 20, 107-118 (2004).
11. Zou, W. Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol 6, 295-307 (2006).
12. Ping Yu, D.A.R., Yang-Xin Fu, Hans Schreiber. The role of stroma in immune recognition and destruction of well-established solid tumors. Curr Opin Immunol 18, 6 (2006).
13. Mueller, N.M., Fusenig, N.E. Friends or foes - bipolar effects of the tumour stroma in cancer. Nat Rev Cancer, 11 (2004).
14. Elenbaas, B., Weinberg, R.A. Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation. Exp Cell Res, 16 (2001).
15. Tuxhorn, J.A., Ayala, G.E., Rowley, D.R. Reactive stroma in prostate cancer progression. J Urol, 14 (2001).
16. Li, G., Satyamoorthy, K., Meier, F., Berking, C., Bogenrieder, T., Herlyn, M. Function and regulation of melanoma-stromal fibroblast interactions: when seeds meet soil. Oncogene, 10 (2003).
17. Garin-Chesa, P., Old, L.J., Rettig, W.J. Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers. Proc Natl Acad Sci U S A, 5 (1990).
18. Park, J.E., Lenter, M.C., Zimmermann, R.N., Garin-Chesa, P., Old, L.J., Rettig, W.J. Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts. J Biol Chem, 8 (1999).
19. Rettig, W.J., Garin-Chesa, P., Beresford, H.R., Oettgen, H.F., Melamed, M.R., Old, L.J. Cell-surface glycoproteins of human sarcomas: differential expression in normal and malignant tissues and cultured cells. Proc Natl Acad Sci U S A, 5 (1988).
20. Huber, M.A., Kraut, N., Park, J.F. Fibroblast activation protein: differential expression and serine protease activity in reactive stromal fibroblasts of melanocytic skin tumors. J Invest Dermatol, 7 (2003).

21. Song, X.T., Evel-Kabler, K.,Shen, L.,Rollins, L.,Huang, X. F.,Chen, S. Y. A20 is an antigen presentation attenuator, and its inhibition overcomes regulatory T cell-mediated suppression. Nat Med 14, 258-265 (2008).
22. Evel-Kabler, K., Song, X.T., Aldrich, M., Huang, X.F. & Chen, S.Y. SOCS1 restricts dendritic cells' ability to break self tolerance and induce antitumor immunity by regulating IL-12 production and signaling. J Clin Invest 116, 90-100 (2006).
23. Song, X.T., Evel-Kabler, K.,Rollins, L.,Aldrich, M.,Gao, F.,Huang, X. F.,Chen, S. Y. An alternative and effective HIV vaccination approach based on inhibition of antigen presentation attenuators in dendritic cells. PLoS Med 3, e11 (2006).
24. Song, X.T., Aldrich, M.,Chen, S. Y. Suppressor of cytokine signaling 1 inhibition strategy to enhance anti-HIV vaccination. Expert Rev Vaccines 5, 495-503 (2006).

Appendices

1. **Abstract:** An A20-silenced, stroma-targeted DC vaccine reverses the immunosuppressive tumor microenviroment resulting in potent antitumor activity. Zhu W, Zhou X, Rollins L, Rooney CM, Gottschalk S, Song XT. Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope Meeting, August 2-5, 2011. Orlando Marriott World Center Hotel, Orlando, FL.

2. **Publication:** Kakarla S, Song XT, Gottschalk S. Cancer-associated fibroblasts as targets for immunotherapy. Immunotherapy. 2012 Nov;4(11):1129-38. doi: 10.2217/imt.12.112. Review.

3. **Manuscript:** Stephen Gottschalk , Feng Yu, Minjun ji, Sunitha Kakarla, Xiao Tong Song. A vaccine that cotargets tumor cells and cancer associated fibroblasts induces bystander T cells with potent antitumor activity

Appendix

An A20-Silenced, Stroma-Targeted DC Vaccine Reverses the Immunosuppressive Tumor Microenviroment Resulting in Potent Antitumor Activity

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Dendritic cell (DC) vaccines have produced disappointing benefits in most clinical studies of cancer. This is most likely due to the presence of immunosuppressive cells within the tumor. Inhibition may come from regulatory T cells (Tregs) and also from the tumor supporting stroma, since cancer associated fibroblasts (CAFs; the central component of the tumor stroma) secrete inhibitory cytokines. Moreover, the extracellular matrix itself may protect malignant cells from effective immunity. We have previously shown that an A20-silenced DC vaccine renders DC resistant to Treg mediated immunosuppression and the aim of this project was to evaluate the effects of an A20-silenced DC vaccine targeting fibroblast activating protein (FAP) expressed on CAFs in the murine 4T1-neu breast cancer model. Bone marrow derived DCs were genetically modified to express an A20-specific shRNA, murine FAP, and rat HER2 (DC-shA20-FAP-HER2). DC-shA20-FAP-HER2 vaccination induced potent CD4+ and CD8+ FAP- and HER2-specific T-cell responses in BALB/c mice as judged by IFN-g Elispot assays. DC-shA20-FAP-HER2 vaccination resulted in potent antitumor effects in prophylactic and therapeutic 4T1-neu models. Vaccinating tumor bearing mice with DC-shA20-FAP induced a significant decrease of tumor-infiltrating macrophages and myeloid-derived suppressor cells in comparison to DC-shA20 vaccinated mice. Conversely, DC-shA20-FAP vaccination induced a 2-fold upregulation of CD80 expression on tumor-infiltrating DCs as well as an increase of infiltrating CD8+ T cells. We show here that an A20-silenced, FAP-targeted DC vaccine induces potent FAP-specific immune response, reverses the immunosuppressive tumor microenvironment and has potent antitumor activity for breast cancer. Thus, targeting the tumor stroma has the potential to improve current DC vaccine approaches for breast cancer.

Keywords: Immunotherapy; Cancer vaccine; Animal Models

A vaccine that cotargets tumor cells and cancer associated fibroblasts induces bystander T cells with potent antitumor activity

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Running Title: Stroma-targeted DC vaccine for cancer immunnotherapy

Keywords: Vaccine, FAP, A20, Melanoma, CAF

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ABSTRACT

Dendritic cell (DC) vaccines have produced limited antitumor activity against bulky tumors in most clinical studies. This is most likely due to the presence of cancer-associated fibroblasts (CAFs), the central component of the tumor stroma, which directly support tumor growth and contribute to the immunosuppressive tumor microenvironment. We have previously shown that inhibiting the antigen presenting attenuator A20 in DCs renders DC resistant to tumor-mediated immunosuppression. In this study we constructed a new compound vaccine, which encodes an A20-specific shRNA (shA20), and targets fibroblast activating protein (FAP) expressed in CAFs and tyrosine-related protein (TRP)2 expressed in melanoma cells (DC-shA20-FAP-TRP2). DC-shA20-FAP-TRP2 vaccination induced robust FAP- and TRP2-specific T-cell responses, resulting in greater antitumor activity in the B16 melanoma model in comparison to monovalent vaccines or a vaccine encoding antigens and a control shRNA. DC-shA20-FAP-TRP2 vaccination enhanced tumor infiltration of CD8-positive T cells, and facilitated cross-presentation of tumor-associated antigens resulting in the induction of bystander, melanoma-specific T cells with potent antitumor activity. Thus, combining A20 inhibition with cotargeting of tumor cells and CAFs in a single vaccine has the potential to improve current vaccine approaches for cancer.

INTRODUCTION

Dendritic cell (DC) vaccines have shown promising antitumor activity in the clinic, highlighted by the FDA approval of a DC vaccine for metastatic castrate resistant prostate cancer (REF). However, bulky tumors are rarely eradicated post vaccination and the majority of patients ultimately progress (REF). This lack of efficacy is most likely due to the presence of immunosuppressive cells within the tumor as well as the tumor supporting stroma (REF) limiting cross presentation and the induction of broad-based tumor antigen-specific T-cell responses.

Cancer associated fibroblasts (CAFs) are the key cellular component of the tumor stroma and are present in the majority of common epithelial cancers such as lung, breast, and prostate cancer, as well as sarcoma and melanoma (REF). CAFs express fibroblasts activation protein (FAP) and the targeted deletion of FAP-positive CAFs in transgenic mouse models has highlighted their central role in tumorigenesis (REF). FAP-based vaccines have antitumor activity in preclinical models and modulate the tumor immune microenvironment (REF). However, anti-FAP induced immune responses were suboptimal, necessitating the use of prophylactic tumor models or the use of chemotherapeutic agents post vaccination in most studies (REF).

We previously have shown that silencing A20, a negative regulator of NF-κb-mediated DC activation, enhances DC function (REF). A20-silenced DCs induced robust cytotoxic and helper T-cell responses *in vivo*, and inhibited regulatory T cells in an antigen-

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specific manner, resulting in enhanced antitumor responses in the B16 ovalbumin (B16-OVA) model (REF).

The intent of this study was now to evaluate a vaccine that silences A20, and cotargets FAP-positive CAFs as well as tumor cells. Our results show that such a compound-vaccine has potent antitumor activity and that cotargeting of CAFs and tumor cells is critical for the induction of cytotoxic T cells specific for unrelated tumor antigens not encoded in the vaccine.

METHODS

Mice. C57BL/6J and Balb/C mice were purchased from Jackson Laboratories and maintained in a pathogen-free mouse facility at BCM according to institutional guidelines. This study was approved by the Institutional Animal Care and Use Committees of BCM.

Lentiviral vector construction, production and transduction. The HIV self-inactivating (SIN) vector used in this study was pSIH1-H1-shRNA vector from SBI. Mouse A20 small hairpin interfering RNA sequence was inserted into pSIH1-H1-shRNA to generate pSIH1-H1-A20-shRNA that contains the A20 shRNA (5'-CTACCTGAGTTCCTTCCCCTTCAAGAGAGGGGAAGGAACTCAGGTAGTTTTT-3'). FAP or TRP-2 cDNA were inserted into pSIH1-H1-shRNA under the control of CMV promoter to generate pSIH1-H1-A20-shRNA-CMV-FAP, pSIH1-H1-A20-shRNA-CMV-TRP2, or pSIH1-H1-A20-shRNA-CMV-FAP-TRP2. Recombinant pseudotyped lentiviral vectors were generated by co-transfection of three plasmids 17 into 293 cells and concentrated by PEG-it™ Virus Precipitation Solution.

DC culture. Bone marrow dendritic cells were obtained as described (Shen et al., 2004a) with the following modifications. Red blood cells were lysed by incubation at room temperature in Red Blood Cell Lysing Buffer (Sigma) and cells were maintained in HyClone RPMI 1640 supplemented with 10% fetal bovine serum (Summit, Fort Collins, CO), non-essential amino acids, HEPES buffer, glutamax, B-Me, IL-4 (20ng/mL), and GM-CSF (20ng/mL, Peprotech) for 5 days. DCs were then transduced with lentivirus for 8 hours, then cultured for an additional 12–16 hours with LPS.

DC immunization and tumor models. DC were washed in PBS and injected in the rear footpad of naïve C57BL/6 or Balb/C mice at 1×10^6 cells/mouse. Mice were sacrificed on

indicated days; inguinal lymph nodes and spleens were removed for intracellular staining and ELISPOT, respectively. For the tumor model, C57BL/6 or Balb/C mice were injected with $5x10^5$ B16 or B16-OVA, or EG7-OVA tumor cells subcutaneously. Five days later, mice were randomly divided into groups (n = 5 per groups) and injected with $1x10^6$ lentivirus-transduced LPS-matured DCs. Tumor volumes were measured two or three times per week with a vernier caliper.

Flow cytometric analysis. Flow cytometric analysis of DCs and T cells and were performed as previously described[18,24]. Inguinal lymph nodes were dissociated and plated in complete RPMI with lentivirus-transduced DCs for an overnight stimulation at 37°C followed by intracellular staining. Stained cells were analyzed on a FACScalibur instrument (BD, Becton Dickinson, Mountain View, CA) using CellQuest software (BD) for all flow-cytometric analyses.

ELISPOT assays. ELISPOT assays of isolated T cells were performed as described previously described[18,24]. spleens were dissociated and purified by using MACS CD4 (L3T4) or CD8 (Ly-2) MicroBeads (Miltenyi Biotec). Cells were plated in triplicate with OT-II or OT-I peptide (10ug/mL) or anti-CD3/CD28 for positive control. Plates were incubated overnight, then IFN-γ secretion was assessed (antibodies: MABTECH). The results were evaluated in a blinded fashion by ZellNet Consulting, Inc. (New York, NY) with an automated ELISPOT reader system, using KS ELISPOT 4.3 software.

51Cr CTL assays. CD8+ CTL responses were assessed with a standard chromium release assay, which measures the ability of in vitro–restimulated splenocytes to lyse target cells (20, 50). Splenocytes pooled from immunized mice were restimulated in vitro in RPMI-1640 containing B16-OVA tumor lysate and IL2 for 4–6 days. B16 and B16-OVA cells were labeled with sodium 51Cr chromate solution for 60 minutes at 37°C with shaking. Different numbers of effector cells were incubated with a constant number of target cells (5 × 104/well) in 96-well U-

bottomed plates (200 μl/well) for 4 hours at 37°C. The supernatants from triplicate cultures were collected. Percent lysis was calculated as (experimental release – spontaneous release) / (maximum release – spontaneous release) × 100.

Statistical analysis. For statistical analysis, we used Student's t-test with a 95% confidence limit, defined as $p < 0.05$. Results are typically presented as means $\pm$ s.e.m. For the bioluminescence experiments, intensity signals were log-transformed and summarized using mean $\pm$ SD at baseline and multiple subsequent time points for each group of mice. Changes in intensity of signal from baseline at each time point were calculated and compared using paired *t*-tests or Wilcoxon signed-ranks test.

RESULTS

Functional characterization of lentiviral vectors encoding shA20, FAP and TRP2

We constructed 4 lentiviral vectors encoding shA20 and FAP (Lv-shA20-FAP), shA20 and TRP2 (Lv-shA20-TRP2), shA20, FAP and TRP2 (Lv-shA20-FAP-TRP2), or a control shRNA, FAP, TRP2 (Lv-shCo-FAP-TRP2) (**Fig. 1A**). Transgene expression, and silencing of A20 was confirmed by RT-PCR in bone-marrow derived DCs transduced with VSV-G pseudotyped lentiviral vectors (**Fig. 1B,C**).

To demonstrate that Lv-transduced DCs induce FAP- and TRP2-specific T-cell responses, mice were vaccinated with $1x10^6$ DC-shA20-FAP, DC-shA20-TRP2, DC-shA20-FAP-TRP2, or DC-shCo-FAP-TRP2. Fourteen days post vaccination CD4- and CD8-positive splenocytes were isolated and the presence of FAP- and TRP2-specific T cells was determined by IFN-γ ELISPOT assays. Mice vaccinated with A20-silenced DC vaccines induced significantly higher FAP- and TRP2-specific T-cell responses ($p < 0.05$) than mice vaccinated with DC-shCo-FAP-TRP2, confirming our previous finding that silencing of A20 in DCs promotes the induction of robust CD4- and CD8-positive antigen-specific T-cell responses *in vivo* (**Fig. 1D,E**). There was no significant difference ($p > 0.05$) in the induction of FAP- or TRP2-specific T cells responses after vaccinating mice with DCs expressing individual (DC-shA20-FAP or DC-shA20-TRP2) or both antigens (DC-shA20-FAP-TRP2), indicating absence of antigenic competition when both antigens are coexpressed.

DC-shA20-FAP-TRP2 vaccine has potent antitumor activity

The B16 melanoma model is ideal to evaluate if targeting FAP-positive tumor stroma enhances antitumor effects since B16 cells do not express FAP (REF). Mice bearing 5 day-old B16 tumors were vaccinated with a single dose of the 1×10^6 DC-shA20-FAP, DC-shA20-TRP2, DC-shA20-FAP-TRP2, or DC-shCo-FAP-TRP2 vaccine. The non-A20 silenced DC vaccine had no antitumor activity where as all A20-silenced vaccines did (**Fig. 2A**). There was no difference between DC-shA20-FAP and DC-shA20-TRP2 vaccines, indicating that the induction of T-cell responses against a stroma antigen like FAP alone can be associated with significant antitumor effects (**Fig. 2A**). Targeting FAP and TRP2 simultaneously with the DC-shA20-FAP-TRP2 vaccine resulted in the greatest antitumor activity (DC-shA20-TRP2 vs DC-shA20-FAP-TRP2, $p<0.05$; DC-shA20-FAP vs DC-shA20-FAP-TRP2, $p<0.05$). This finding was confirmed using a DC-shA20-FAP-OVA vaccine in B16-OVA and EG7-OVA models (**Supplemental Fig. 1A,B**).

DC-shA20-FAP-TRP2 vaccine increases FAP- and TRP2-specific CD8-positive T cells in tumors

To investigate the mechanisms of how the DC-shA20-FAP-TRP2 vaccine enhances antitumor effects, we first determined the frequency of CD4- and CD8-positive T cells in B16 tumors 3 weeks post vaccination. While there was no significant difference in the percentage of CD4-positive T cells within tumors between groups of vaccinated mice (data not shown), there was a significant ($p<0.05$) 4-fold increase in the percentage of

CD8-positive T cells after DC-shA20-FAP-TRP2 vaccination in comparison to control mice (**Fig. 3A,B**). In contrast, the monovalent vaccines or the non-silenced FAP/TRP2 DC vaccine did not increase the frequency of CD8-positive T cells. To investigate the specificity of the infiltrating CD8-positive T cells, tumor-infiltrating lymphocytes (TILs) were isolated 3 weeks post vaccination. Intracellular cytokine staining for IFN-γ was performed post stimulation with FAP or TRP2 expressing DCs. DC-shA20-FAP-TRP2 vaccination induced the highest frequency of FAP- and TRP2-specific T cells in comparison to the other vaccines (**Fig. 3C**), mirroring the previously observed overall increase in the number of CD8-positive T cells within tumors.

DC-shA20-FAP-TRP2 vaccine induces bystander T-cell responses by activating APCs within tumors

In the previous experiment, the DC-shA20-FAP vaccine induced TRP2-specific T-cell responses (**Fig. 3C**), suggestive of epitope spreading and the induction of bystander T-cell responses. To further investigate this finding, B16 tumor-bearing mice were vaccinated on day 5 with DC-shA20-FAP, DC-shA20-TRP2, DC-shA20-FAP-TRP2, or PBS. After 3 weeks the frequency of T cells specific for the B16-associated tumor antigens tyrosinase-related protein 1 (TRP1), tyrosinase (Tyr), gp100, and melanoma-associated antigen recognized by T cells (MART1) was determined in splenocytes as well as tumor infiltrating lymphocytes. Only the DC-shA20-FAP-TRP2 vaccine induced systemic T-cell responses against TRP1, Tyr, gp100, and MART1 (**Fig.4 A**). While all vaccines induced a significant increase of TRP1-, Tyr-, gp100-, and MART1-specific T cells in TILs in comparison to PBS-injected mice (**Fig. 4B**), the frequency of melanoma-

specific T cells was highest in DC-shA20-FAP-TRP2 vaccinated mice (p<0.05). These results indicate that targeting of the tumor stroma as well as the tumor is necessary to induce potent bystander, tumors-specific T-cell responses.

To investigate the mechanisms of enhanced bystander T-cell induction we determined the expression of the costimulatory molecules CD80 and CD86 on F4/80-positive (monocytes/macrophages) and CD11c-positive (DCs) APCs within tumors, and their ability to cross present the B16-associated antigen gp100. DC-shA20-FAP, DC-shA20-TRP2, and DC-shA20-FAP-TRP2 vaccines induced the expression of CD80 and CD86 on intratumoral APCs in comparison to PBS-vaccinated mice (**Fig 5A**). While monocytes/macrophages and DCs isolated form tumors of DC-shA20-FAP-TRP2 vaccinated mice had the highest level of CD80/CD86 expression, this difference did not reach statistical significance. To evaluate if monocytes/macrophages or DCs, isolated from tumors of DC-shA20-FAP, DC-shA20-TRP2, or DC-shA20-FAP-TRP2 vaccinated mice, were able to cross-present gp100 we performed IFN-γ ELISPOT assays using freshly isolated APCs from tumors and splenocytes from pmel17/gp100 TCR transgenic mice (gp100-specific TCR T cells) as effectors. Only APCs isolated from tumors of DC-shA20-FAP-TRP2 vaccinated mice were able to activate gp100-specific T cells (**Fig. 5B**), indicating their ability to cross-present antigens released from dying B16 melanoma cells.

To provide direct evidence that a stroma/tumor-targeted vaccine facilitates cross-presentation we took advantage of the MHC-OVA$_{(257-264)}$ antibody, that recognizes the

SIINFEKL peptide in the context of MHC class I. B16-OVA bearing mice were vaccinated on day 5 with DC-shA20-FAP, DC-shA20-OVA, or DC-shA20-FAP-OVA, and after 3 weeks the presence of SIINFEKL peptide/MHC class I complexes on the cell surface of monocytes/macrophages or DCs was determined by FACS analysis. Only APCs isolated from tumors of DC-shA20-FAP-OVA vaccinated mice had an increase of SIINFEKL/MHC class I complexes on their cell surface (**Fig. 5C**), confirming that cotargeting of the tumor cells and CAFs is necessary for effective cross-presentation of tumor-derived antigens by intratumoral APCs.

Induced bystander T cells have antitumor activity

Having shown that the developed compound vaccines induced B16-specific, bystander T cells we finally wanted to determine if these T cells have anti-tumor activity. Mice were inoculated with B16-OVA and B16 on their right or left flanks, and after 5 days vaccinated with DC-shA20-FAP, DC-shA20-OVA, DC-shA20-FAP-OVA, or PBS. DC-shA20-OVA vaccination only inhibited the growth of B16-OVA, while the DC-shA20-FAP vaccine inhibited B16 as well as B16-OVA tumor growth (**Fig. 6A,B**). The DC-shA20-FAP-OVA vaccine had the greatest antitumor activity against both cell lines ($p < 0.05$; **Fig. 6A,B**).

To further demonstrate the systemic induction of cytotoxic bystander T-cell responses in this model, mice were injected with B16-OVA and vaccinated after 5 days with DC-shA20-FAP, DC-shA20-OVA, DC-shA20-FAP-OVA, or PBS. After 3 weeks splenocytes of vaccinated mice were isolated and restimulated for 5 days *ex vivo* before performing

a cytotoxicity assay with B16-OVA and B16 cells as targets. Splenocytes harvested from DC-shA20-FAP or DC-shA20-FAP-OVA vaccinated mice showed significant killing of B16-OVA and B16 cells (**Fig. 6C,D**), where as the DC-shA20-OVA vaccine primarily induced OVA-specific T-cell responses.

DISCUSSION

Our results demonstrate that a vaccine that inhibits the antigen presenting attenuator A20, and targets FAP-positive CAFs and the tumor antigen TRP2 has potent antitumor effects, enables cross-presentation resulting in the broad-based induction of T cells specific for tumor antigens not included in the vaccine.

FAP-positive CAFs have emerged as key players in promoting tumor growth of solid tumors (REF). For example, in transgenic mice, in which the diphtheria toxin receptor is expressed under the control of the FAP promoter, administration of diphtheria toxin, which does not kill tumor cells, results in complete ablation of solid tumors (REF). FAP is a membrane bound aminopeptidase, which is involved in remodeling of the extracelluar matrix of tumors. Inhibition of the aminopeptidase activity of FAP resulted in decreased tumor growth and was associated with an accumulation of collagen, decreased numbers of myofibroblasts, and decreased tumor vascularization (REF). Targeting FAP with monoclonal antibodies has been less successful. While a humanized FAP antibody, sibrotuzumab, preferentially localized to metastatic tumor sites after administration to humans, no antitumor effect was observed.

Several groups have conducted vaccine studies targeting solely FAP, demonstrating that S. typhimuirum-, plasmid-, or DC-based FAP vaccines have antitumor activity in the preventive or early therapeutic setting, modulate the tumor microenvironment by promoting Th1 polarization, and enhancing the infiltration of CD8-positive T cells. While

cotargeting of FAP-positive CAFs and TRP2 with a DC vaccine was evaluated in a single set of experiments in the B16-model by one group of investigators, showing a significant delay in tumor growth, no mechanistic studies were performed .

Our studies now extend these findings and demonstrate that silencing the ubiquitin ligase A20, an antigen presenting attenuator in DCs, results in a significant increase in the frequency of FAP-specific T cells. We observed no difference in the antitumor activity of DC-shA20-FAP and DC-shA20-TRP2, indicating that targeting CAFs by FAP vaccination can induce antitumor effects similar to vaccines that target malignant cells. Vaccination with DC-shA20-FAP-TRP2 resulted in the greatest antitumor activity. Mechanistic studied revealed that cotargeting of CAFs and tumor cells resulted in cross-presentation resulting in the induction of bystander, melanoma-specific T cells with potent antitumor activity.

Vaccine studies in breast cancer and renal carcinoma patients have shown that cross-presentation and epitope spreading correlates with improved overall survival. In addition, the induction of broad-based tumor-specific T-cell responses after adoptive T-cell transfer in one melanoma patient resulted in a complete response. Currently, the determinants of effectively inducing cross-presentation and epitope spreading are unknown. Our study indicates that the destruction of CAFs and tumor cells is critical for this process. Importantly, the induced bystander T cells were cytotoxic and had antitumor activity against antigen-loss variants, which have been reported in humans

enrolled on vaccine and T-cell immunotherapy studies that only targeted tumor antigens.

In summary, the compound vaccine we describe here induces CAF- and tumor-specific immune responses and elicits broad-based T-cell responses against tumor antigens. Thus, targeting CAFs in addition to malignant cancer cells has the potential to improve current DC vaccine approaches for cancer.

Acknowledgements

FIGURES LEGENDS

Figure 1: Tumor- and FAP- co-targeted DC induce T-cell activation. (A) Scheme of lentiviral constructs. **(B and C)** Mouse BM-DCs were transduced with lentivirus and FAP and TRP2 expression **(B)** and A20 expression **(C)** were detected by RT-PCR or Q-PCR individually. **(D and E)** Mice were immunized with 1 × 10^6 lentivirus-transduced BM-DCs in 25 µl sterile PBS or PBS control through footpad. 14 days post vaccination splenocytes were prepared and CD8+ **(D)** and CD4+ **(E)** T cells selected. The frequency of FAP- and TRP2-specific T cells was determined using IFN-γ ELISPOT assays. AF, lentiviral vector coexpressing an A20-specific short-hairpin RNA (shRNA) and FAP; AT, lentiviral vector coexpressing A20-shRNA and TRP2; AFT, lentiviral vector coexpressing A20-shRNA, FAP, and TRP2; CFT, lentiviral vector coexpressing GFP-shRNA, FAP, and TRP2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PBS, phosphate-buffered saline.

Figure 2: DC-shA20-FAP-TRP2 vaccine has potent antitumor activity. Mice were inoculated with B16 followed by immunization with 1x10^6 DC-shA20-FAP (AF), DC-shA20-TRP2 (AT), DC-shA20-FAP-TRP2 (AFT), DC-shCo-FAP-TRP2 (ACT) or PBS on day 5 (n= 5 per group). Cotargeting FAP and TRP2 with AFT resulted in the greatest antitumor response (AF vs AFT, p<0.05; AF vs AFT, p<0.05).

Figure 3: DC-shA20-FAP-TRP2 vaccine increases FAP- and TRP2-specific CD8-positive T cells in tumors. Mice were inoculated with B16 tumors followed by

immunization with 1x10^6 DC-shA20-FAP (AF), DC-shA20-TRP2 (AT), DC-shA20-FAP-TRP2 (AFT) or PBS on day 5. Tumor tissues were dissected 3 weeks post vaccination (n=5). **(A)** Representative FACS analysis of infiltrating CD8+ T cells. **(B)** Summary data of all mice showing a significant increase (p<0.05) of CD8+ T cells in mice vaccinated with AFT. **(C)** TILs were restimulated with DCs transduced with FAP (DC-Lv-FAP) or TRP2 (DC-LV-TRP2) followed by intracellular staining of IFN-γ. The experiment has been repeated twice with similar results.

Figure 4: DC-shA20-FAP-TRP2 vaccine activates bystander T cells. Mice were inoculated with B16 tumors followed by immunization with 1x10^6 DC-shA20-FAP (AF), DC-shA20-TRP2 (AT), DC-shA20-FAP-TRP2 (AFT) or PBS on day 5. Splenocytes and TILs were prepared 3 weeks post vaccination. **(A)** Splenocutes were subjected to IFN-γ ELISPOT assays. DC-Lv-TRP2, DC-Lv-TRP1, DC-Lv-TYR, DC-Lv-gp100, DC-Lv-MART1 or mock transduced DCs (PBS) were used as APCs. There was a significant increase (p<0.05) of bystander T cells specific for TRP1, TYR, gp100, and MART1 only in AFT vaccinated mice. The experiment has been repeated twice with similar results. **(B)** TILs were restimulated with DC-Lv-TRP2, DC-Lv-TRP1, DC-Lv-TYR, DC-Lv-gp100, DC-Lv-MART1 or mock transduced DCs (PBS) followed by intracellular staining of IFN-γ. FACS analysis and a cumulative bar graph are shown. TILs isolated from AFT vaccinated mice had the highest frequency of B16-speciifc T cells (p<0.05). The experiment has been repeated twice with similar results.

Figure 5: DC-shA20-FAP-TRP2 vaccine activates APCs within the tumor. (A and B) Mice were inoculated with B16 tumors followed by immunization with 1x10^6 DC-shA20-FAP (AF), DC-shA20-TRP2 (AT), DC-shA20-FAP-TRP2 (AFT) or PBS on day 5. 3 weeks post vaccination the expression of CD80 and CD86 on intratumoral monocytes/macrophages (F4/80) and DCs (CD11c) was determined. The experiment has been repeated twice with similar results. **(B)** Intratumoral monocytes/macrophages (CD11b) and DCs (CD11c) were isolated and used as APCs in IFN-γ ELISPOT assays using gp100-specific T cells as effectors. Only APCs isolated from tumors of mice vaccinated with AFT were able to activate gp100-specific T cells. The experiment has been repeated twice with similar results. **(C)** Mice were inoculated with B16-OVA followed by immunization with 1x10^6 DC-shA20-FAP (AF), DC-shA20-OVA (AO), DC-shA20-FAP-OVA (AFO) or PBS 5 days later. 3 weeks later the presence OVA peptide/MHC calls I complexes was determined on APCs (F4/80+ and CD11c+ cells) by FACS analysis. The experiment has been repeated twice with similar results.

Figure 6: Induced bystander T cells have antitumor activity. (A and B) Mice were inoculated with B16-OVA or B16 tumor on the right or left flank separately followed by immunization with 1x10^6 DC-shA20-FAP (AF), DC-shA20-OVA (AO), DC-shA20-FAP-OVA (AFO) or PBS 5 days later (n= 5 per group). B16-OVA **(A)** and B16 **(B)** tumor growth was measured. The AFO and the AF vaccine had anti-B16 activity with AFO being superior (p<0.05). **(C and D)** Mice were inoculated with B16-OVA followed by immunization with 1x10^6 AF, AO, AFO, and PBS 5 days later. 3 weeks later, splenocytes were prepared and cultured in vitro in the presence of B16-OVA tumor
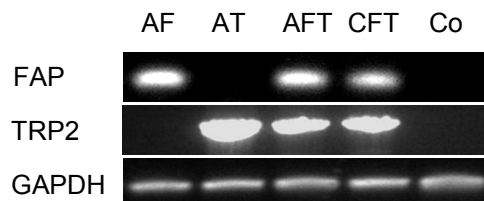
lysate and IL2 for 5 days and their cytolytic activity was evaluated in standard subjected ^{51}Cr release assay against B16-OVA **(C)** or B16 **(D)**. Splenocytes isolated from AF and AFT vaccinated mice had significant cytotoxic activity B16. (* AFO vs AF & AO, p<0.05; ** AF & AO vs PBS, p<0.05;.*** AFO & AF vs AO, p<0.05.).

Supplemental Figure 1: DC-shA20-FAP-OVA vaccine has potent antitumor activity. Mice were inoculated with B16-OVA **(A)** or EG7-OVA **(B)** followed by immunization with 1x10^6 DC-shA20-FAP (AF), DC-shA20-OVA (AO), DC-shA20-FAP-OVA (AF) or PBS on day 5 (n= 5 per group). Cotargeting FAP and OVA with AFO resulted in the greatest antitumor activity in both models (B16-OVA: AF vs AFT, AF vs AFT, p<0.05; EG7-OVA: AF vs AFT, p<0.05; AF vs AFT, p<0.05;).

Figure 1

A

B

C

D

Figure 2

Figure 3

A

PBS | CFT | AF

AT | AFT

FCS

CD8-APC

B

*p<0.05

% of CD8+ tumor infiltrating cells

0.22   0.28   0.23   0.27

0.88

PBS   CFT   AF   AT   AFT

C

PBS | CFT | AF | AT | AFT

1.21 | 9.95 | 17.22 | 10.50 | 23.13   TRP2

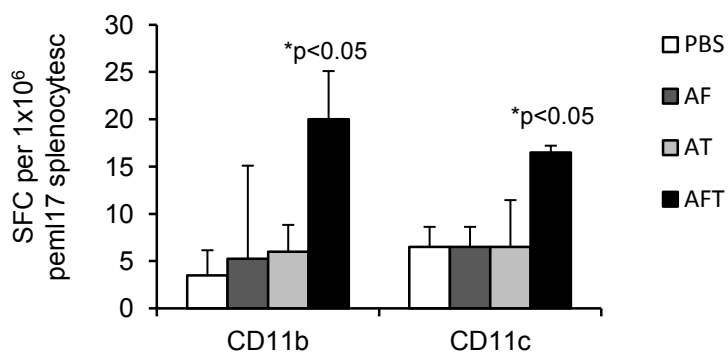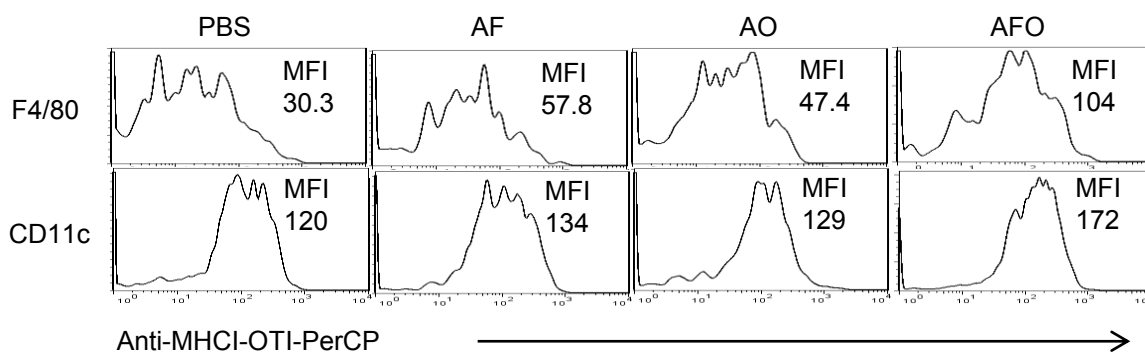0.93 | 4.38 | 8.82 | 2.31 | 11.91   FAP

IFN$_\gamma$-PE

CD8-APC

Figure 4

Figure 5

A
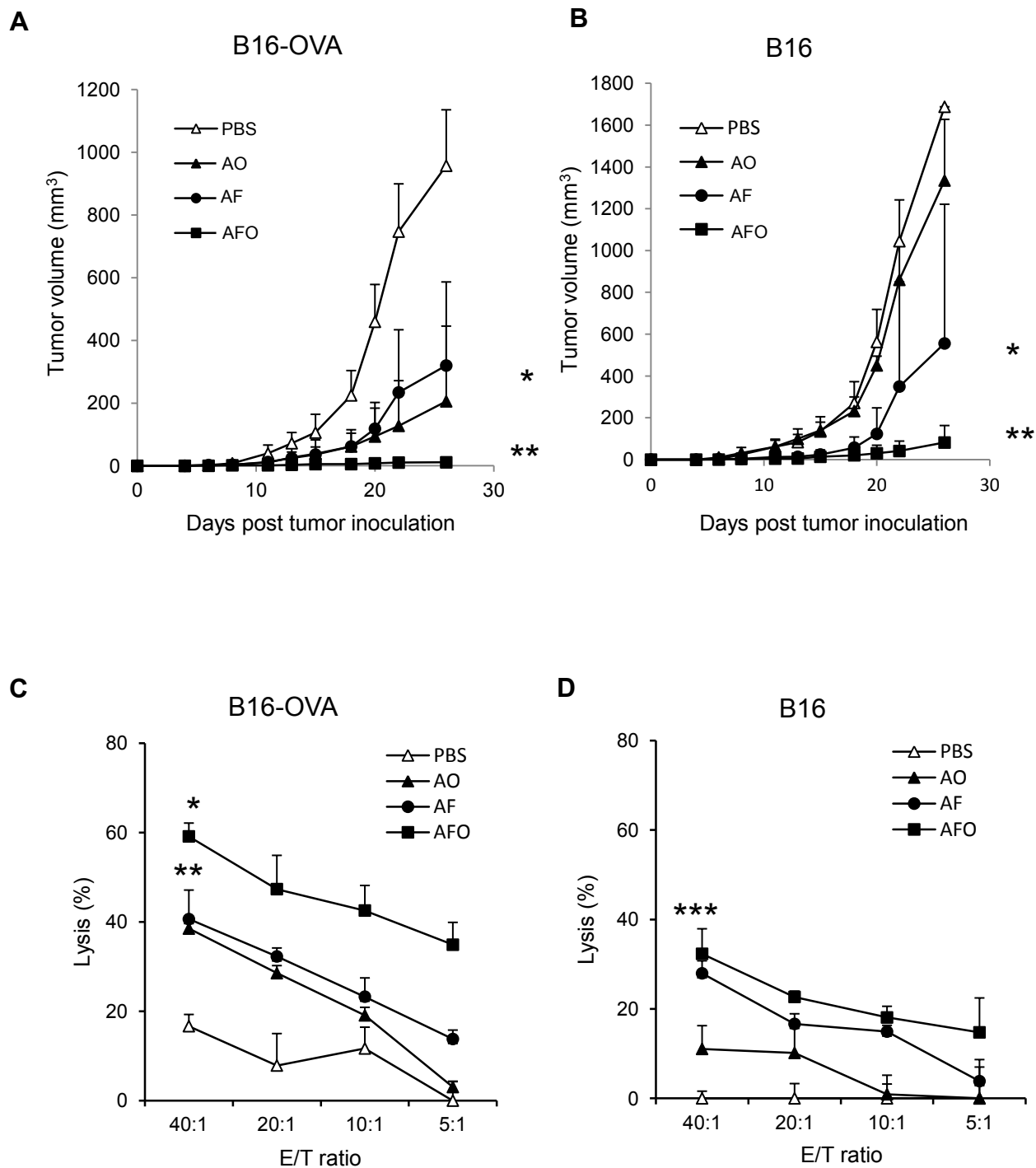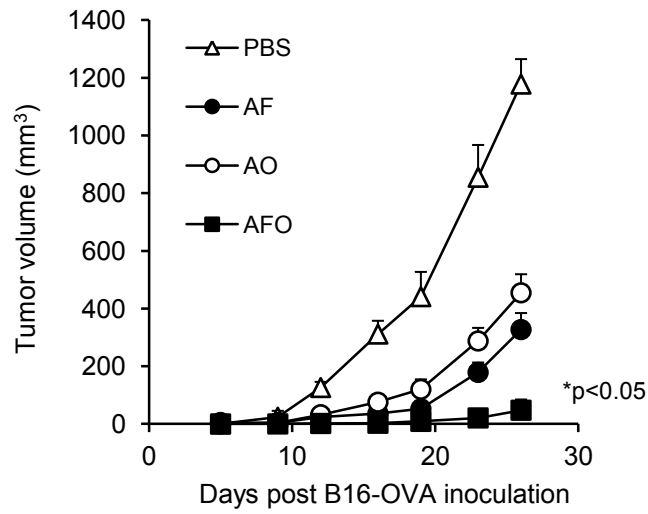

B

C

Figure 6

A

B16-OVA

B

B16

C

B16-OVA

D

B16

A

B